Loss-of-Function Mutations in *CAST* Cause Peeling Skin, Leukonychia, Acral Punctate Keratoses, Cheilitis and Knuckle Pads (PLACK) Syndrome

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ABSTRACT

Calpastatin is an endogenous specific inhibitor of calpain, a calcium-dependent cysteine protease. Here we show that loss-of-function mutations in the calpastatin gene (*CAST*) are the genetic cause of an autosomal recessive condition characterized by generalized peeling skin, leukonychia, acral punctate keratoses, cheilitis and knuckle pads (PLACK syndrome). In affected individuals with PLACK syndrome from three families of different ethnicities, we identified homozygous mutations (c.607dup, c.424A>T and c.1750delG) in *CAST*, all of which were predicted to encode truncated proteins (p.Ile203Asnfs*8, p.Lys142* and p.Val584Trpfs*37). Immunocytochemistry shows that expression of calpastatin is reduced in skin from affected individuals. Transmission electron microscopy revealed widening of intercellular spaces with chromatin condensation and margination in the upper stratum spinosum in lesional skin, suggesting impaired intercellular adhesion as well as keratinocyte apoptosis. A significant increase of apoptotic keratinocytes was also observed in TUNEL assays. *In vitro* studies utilizing siRNA-mediated *CAST* knockdown revealed a role for calpastatin in keratinocyte adhesion. In summary, we describe PLACK syndrome, as a novel clinical entity of defective epidermal adhesion, caused by loss-of-function mutations in the *CAST* gene.

MAIN TEXT

Peeling skin syndrome (PSS) is characterized by continuous shedding of the stratum corneum of the epidermis with onset from birth or infancy and lasting throughout life.[1] Skin peeling may be accompanied by erythema, vesicular lesions, or other ectodermal features including fragile hair and nail abnormalities.[2] PSS can be divided into two clinical forms: acral PSS (APSS, MIM 609796) and generalized PSS (GPSS, MIM 270300). APSS involves the palmar, plantar and dorsal surfaces of hands and feet and can be associated with mutations in the transglutaminase 5 gene (*TGM5*, MIM 603805).[3] In addition, mutations in the gene encoding for the cysteine protease inhibitor cystatin A (*CSTA*, MIM 184600) have recently been associated with autosomal recessive exfoliative ichthyosis (MIM 607936) and also APSS.[4,5] Individuals with inflammatory GPSS associated with mutations in the corneodesmosin gene (*CDSN*, MIM 602593) can also present with severe pruritus, food allergies and repeated episodes of angioedema, urticaria, and asthma.[6] A homozygous missense mutation was identified within the *CHST8* gene (MIM 610190), encoding N-acetylgalactosamine-4-O-sulfotransferase, in a large consanguineous family with non-inflammatory GPSS.[7] However, the genetic basis of a number of PSS cases is still unresolved.[8,9]

Here we show that homozygous loss-of-function (LOF) mutations in the *CAST* gene underlie autosomal recessive generalized PSS with leukonychia, acral punctate keratoses, cheilitis and knuckle pads. We propose this novel clinical entity to be given

the acronym PLACK syndrome. Using exome sequencing and Sanger sequencing we demonstrate that distinct homozygous LOF *CAST* gene mutations segregate with the disorder in all three families with PLACK syndrome. *CAST* encodes calpastatin, an endogenous protease inhibitor. Our findings emphasize the important role of the protease-inhibitor balance in epidermal homeostasis.

We ascertained a 28-year-old Chinese female (individual 1) affected with PLACK syndrome who was born to second-cousin parents (Figure 1A). She was found to have trauma-induced recurrent blistering prominently on the extremities since infancy, which was worse in summer. In winter, asymptomatic skin peeling was more prominent, either spontaneously or following the remission of blistering, leaving underlying erythema. The blistering improved and was confined to distal extremities after puberty, while skin peeling progressed to involve nearly the entire body. She had mild pruritus and no history of atopy. Physical examination revealed generalized dry, scaly skin with superficial exfoliation and underlying erythema (Figure 1B). Cheilitis with dry, shedding scales was observed (Figure 1B). Several blisters were noted on her wrists and soles. Punctate palmoplantar keratoderma was seen, which coalesced into focal keratoderma predominately on the weight-bearing areas (Figure 1B). Knuckle pads with multiple hyperkeratotic micropapules were also found involving all the interphalangeal joints. Leukonychia affected the proximal half of the nails with mild distal onycholysis (Figure 1B). No other systemic abnormalities were identified. Histological examination of a biopsy from the scaly skin of her leg showed

hyperkeratosis, acanthosis, intraepidermal clefting with irregular acantholysis (Figure 1C).

We also investigated an affected Nepalese female (individual 2) from non-consanguineous parents who presented to dermatologists at age 3 with a 2-year history of painful lesions on the palms and soles. Clinical examination revealed punctate keratoses on the palms, extending onto the flexor aspect of the wrists and soles, plaques with hyperkeratotic micropapules over the interphalangeal joints, cheilitis of the upper lip and angular cheilitis, subtle telangiectasia on the cheeks, follicular hyperkeratosis on the extensor surface of the knees and leukonychia of 70-100% of the nails (Figure 1D and Figure S1, available online). At review at age 4, peeling areas had developed on the distal limbs including the extensor surface of the knees and elbows (Figure 1D).

An additional family with known consanguinity, previously reported as having a recessive form of pachyonychia congenita, was also enrolled in this study.[10] Briefly, both affected Caucasian male siblings (individuals 3 and 4, now age 54 and 58 years old) give a history of blistering and peeling of skin from the age of about 3 months on the hands, feet, knuckles, elbows and knees. Skin fragility is induced by minor trauma and heat and continues to be the greatest problem for these individuals. Both individuals also have leukonychia, leukokeratosis, angular cheilitis, papules on the extensor surface of the fingers and toes and punctate palmar keratosis and a plantar

keratoderma, described in more detail by Haber & Rose.[10]

This project was approved by the Clinical Research Ethics Committee of the Peking University First Hospital, East London and City Health Authority Research Ethics Committee and the Western Institutional Review Board which all comply with all principles of the Helsinki Accord.

We collected blood and saliva samples from the three families after obtaining informed consent. After exclusion of pathogenic mutations in *TGM5*, *CSTA*, *CDSN* and *CHST8* by Sanger sequencing, we performed exome sequencing in individual 1 using 3 μg of genomic DNA. Exome capture was performed with the Nimblegen SeqCap EZ Library (Roche) for enrichment and then sequenced on an Illumina HiSeq2000 according to the manufacturer's protocols. Variants were filtered against dbSNP137, the 1000 Genomes Project, HapMap8 and BGI inner databases, as described in our previous study.[9] As her parents were consanguineous, we focused on homozygous variants to identify the causative gene. Among all the 148 variants fulfilling these criteria, only 15 variants lay in coding exons or splicing boundaries and were predicted to be "not tolerated" by SIFT.[11] These included a one-nucleotide duplication in *CAST* which was predicted to lead to a frameshift and truncation of the protein. By Sanger sequencing (primers are listed in Table S1, available online), we confirmed that this mutation c.607dup (p.Ile203Asnfs*8; RefSeq accession number NM_001042440.3, NP_001035905.1) was homozygous in individual 1 and

heterozygous in her unaffected parents, siblings and daughters (Figures 1A and 2A). This mutation was not found in 200 ethnically matched controls.

Exome sequencing was performed on genomic DNA from individual 2 using the NimbleGen™ SeqCap EZ Library SR (Roche) and sequenced on an Illumina HiSeq2000 according to the manufacturer's protocols. Further details of analysis are described previously.[11] Analysis of novel variants revealed a homozygous nonsense mutation c.424A>T (p.Lys142*) in *CAST*. Sanger sequencing showed co-segregation with the disorder in this family (Figure 2A and Figure S1A). Subsequent exome sequencing and Sanger sequencing also showed a homozygous frameshift mutation c.1750delG (p.Val584Trpfs*37) in *CAST* in individuals 3 and 4, which co-segregated with the disorder in the family (primers are listed in Table S1).

All three mutations identified in *CAST* are predicted to encode truncated proteins and lead to a loss of function. To investigate the consequences of homozygosity for the c.607dup mutation *in vivo*, we examined the mRNA expression of *CAST* in the skin from individual 1 by using quantitative real-time PCR (qRT-PCR) (primers are listed in Table S2). The level of *CAST* transcription was determined on the basis of the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method using cDNA templates generated from a corresponding area of the skin from a gender and age-matched healthy control as the calibrator. In contrast to the control, we could only detect trace expression of *CAST* mRNA in individual 1, probably due to mechanisms of nonsense-mediated

mRNA decay (Figure 2B). Immunohistochemical staining was subsequently performed using a calpastatin antibody (catalog number: sc-20779, Santa Cruz) in paraffin-embedded sections from the pretibial skin lesion of individual 1. A similar analysis was performed on paraffin-embedded sections taken from xerotic left thigh skin of individual 2, but using immunofluorescence. Consistent with the predicted effect of the two mutations and the results of the qRT-PCR analysis of the c.607dup mutation, calpastatin expression was absent/reduced in the skin from both individuals, whereas in the normal control skin calpastatin is localized throughout the epidermis and has a cytoplasmic localization (Figure 2C and Figure S2). No skin biopsy material was available from individuals 3 or 4.

Calpastatin is a specific endogenous protease inhibitor of calpains (μ-calpain and m-calpain). Calpains are intracellular cysteine proteases that require calcium or epidermal growth factor for their catalytic activity.[12] In skin, calpains have been reported as being involved in a range of cellular processes, including keratinocyte growth, migration and cell cycle regulation.[13] These processes are particularly important in epidermal terminal differentiation, characterized by expression of specific structural proteins. Previous studies have shown that calpains can degrade filaggrin to peptides.[14] Immunohistochemical staining of lesional skin sections from individual 1 demonstrated that filaggrin expression (antibody catalog number: sc-66192, Santa Cruz) was markedly reduced compared to control (Figures 3A and 3B). The degradation of filaggrin could explain, at least in part, the ichthyosiform

cutaneous changes. A slight upregulation of loricrin (antibody catalog number: ab24722, Abcam), keratin 1 (antibody catalog number: ab24643, Abcam) and keratin 10 (antibody catalog number: ab76318, Abcam) was also observed in the lesional skin from individual 1 (Figure S3). The increased expression of these proteins may be a compensatory mechanism for epidermal barrier disruption.[15]

As increased activity of calpains can induce apoptosis,[16] *in situ* apoptotic examination of keratinocytes in the lesional skin biopsy from individual 1 was performed using a TUNEL assay (*In Situ* Cell Death Detection Kit, Roche). Skin sections from an unrelated healthy subject were used as a control. Images above the stratum basale were taken randomly with a fluorescence microscope (IX71, Olympus, Tokyo, Japan), using the same settings for both control and individual skin sections. Total cells and apoptotic cells (with fluorescent nuclei) in five random high-power fields (400X) were counted. The number of apoptotic cells in the imaged fields was significantly increased in lesional skin compared to normal control (Figures 3C and 3D). Ultrastructural analysis of lesional skin sections from individual 1 was performed by transmission electron microscope (TEM), which showed expanded intercellular spaces (Figure 3E), apoptotic chromatin condensation and margination (Figure 3F), supporting the results of the TUNEL assay.

To determine the functional consequences of *CAST* LOF mutations *in vitro*, we performed siRNA-mediated knockdown (KD) of *CAST* using a specific siRNA pool

(ON-TARGETplus Human CAST siRNA-SMARTpool, GE Healthcare Dharmacon) in the immortalized keratinocyte cell line, HaCaT. Non-targeting pool siRNA (ON-TARGETplus Non-targeting Pool, GE Healthcare Dharmacon) was used as a control. Immunocytochemistry on HaCaT cell monolayers treated with *CAST* siRNA and analysis of total protein lysates by western blotting showed robust CAST knockdown (Figures 4A-4C).

We then used an *in vitro* mechanical-induced stress assay to investigate the role of calpastatin in keratinocyte adhesion in *CAST* siRNA-treated cells (CAST KD cells) and NTP siRNA-treated cells (NTP cells). For this we have used the Flexcell FX-4000 Tension System (Flexcell, Hillsborough, NC) which uses vacuum pressure to apply cyclic or static strain to cells cultured on flexible-bottomed culture plates. CAST KD cells (mimicking the homozygous LOF mutations) and NTP cells were subjected to mechanical stretch at a frequency of 5 Hz (five cycles of stretch and relaxation per second) and an elongation of amplitude ranging from 10% to 14% (increase in diameter across the silicone deformable membrane from 10% to 14%). Cells were stretched for 0 hr (non-stretched) and 4 hr. Immunocytochemistry with an in-house LL001 monoclonal keratin 14 antibody[17] on siRNA-treated cells pre- and post-mechanical stress revealed breakage of the intercellular connections in CAST KD cell monolayers independent of whether they had been subjected to mechanical stress. In contrast, NTP cells presented with stretched keratin filaments post-stretching but no disruption in intercellular adhesion prior to mechanical stress (Figures 4D-4G).

A recent study by Nassar et al. looking at calpastatin overexpression in a mouse model reported significant changes in the wound-healing process compared to normal mice.[18] Calpastatin overexpression mice showed a striking delay in wound-healing with reduced proliferation and re-epithelialization, particularly in the early steps of the wound-healing process.[18] The possible effects of *CAST* LOF mutations on keratinocyte migration were assessed by performing a scratch assay on CAST KD cell monolayers compared to NTP cells. As CAST KD scratch-wounds appeared to close at the same rate as NTP scratch-wounds, we concluded that cell migration in monolayers was not altered by CAST knockdown in 3 independent experiments (Figure S4).

Following observations in *CAST* LOF skin and our *in vitro* studies indicating a key role for calpastatin in keratinocyte adhesion, we examined desmosomal protein expression. Immunofluorescence with an antibody targeting Desmoplakin (DSP; 11-5F mouse monoclonal, a gift from David Garrod),[19] the major protein of the desmosome, was performed on frozen skin sections from individuals 1 and 2. This showed an apparent increase in DSP expression, with both a plasma membrane and cytoplasmic localization pattern compared to a specific membranous localization pattern in control skin (Figures 4H and 4I and Figures S3G and S3H). Furthermore, our *in vitro* studies displayed a general trend of DSP upregulation in CAST KD cells independent of mechanical stress when compared to NTP cells (data not shown).

Calpain and its endogenous specific inhibitor calpastatin constitute an intracellular non-lysosomal proteolytic system ubiquitously expressed in mammals and many other organisms. By catalyzing the controlled proteolysis of target proteins, calpains play an important role in various cell functions, including cell proliferation, differentiation, mobility, cell cycle progression, as well as cell-type specific functions like cell fusions in myoblasts.[20,21] Also, activation of calpains has been suggested to trigger apoptosis by cleaving either pro-apoptotic or anti-apoptotic proteins.[22] It has been demonstrated *in vitro* that increased activity of m-calpain results in apoptosis of HaCaT cells.[23] LOF mutations in *CAST* lead to disinhibition of calpains, thus enhancing apoptosis of keratinocytes, as showed in our TUNEL and TEM results. Elevated apoptotic levels of keratinocytes may result in skin hyperkeratosis,[24,25] leading to the clinical phenotypes of acral punctate keratoses, knuckle pads with hyperkeratotic micropapules and leukonychia.

Previous studies have shown that calpain could promote focal adhesion disassembly by proteolysis of talin and focal adhesion kinase.[26,27] Our CAST KD experiments indicate that the calpain/calpastatin system may also be critical for intercellular adhesion. Increased calpain activity due to LOF mutations in *CAST* may lead to excessive proteolysis of epidermal desmosomal components, as demonstrated by aberrant expression of DSP in the affected individuals, resulting in acantholysis and impaired resistance of the epidermis to mechanical stretch (blistering and skin

peeling). Although calpastatin was suggested to play diverse physiological roles in neurological, musculoskeletal and ocular systems,[28] no significant related symptoms were observed in our affected individuals. Further studies are required to elucidate whether there is redundancy of the calpain proteolytic system in these organs, explaining the lack of abnormalities. Notably, *Cast*-knockout mice, which showed increased activity of calpains exhibit no defect under normal conditions[29] but only slight behavioral changes in a stressful environment.[30] These phenotypic differences indicate different physiological functions of the calpain/calpastatin system between humans and mice.

In summary, we describe the clinical features of a new autosomal recessive entity termed PLACK syndrome with generalized skin peeling, leukonychia, acral punctate keratoses, cheilitis and knuckle pads, distinct from epidermolysis bullosa or pachyonychia congenita. In three families with this condition, homozygous LOF mutations in *CAST* were identified leading to reduced expression of calpastatin, the only known inhibitor of calpains. Mutations of protease inhibitors can disrupt the skin barrier, impair keratinocyte adhesion, affect cell signaling and cause various genetic skin conditions,[4,31] such as Netherton syndrome (MIM 256500) caused by mutations in *SPINK5* (MIM 605010),[32] Nagashima-type palmoplantar keratosis (MIM 615598) caused by mutations in *SERPINB7* (MIM 603357).[33,34] Our findings expand the spectrum of these conditions and explore new avenues for proteolytic pathways in skin.

Supplemental Data

Supplemental data include 2 tables and 4 figures and can be found with this article online at http://www.cell.com/AJHG/.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes project, http://www.1000genomes.org/

dbSNP, http://www.ncbi.nlm.nih.gov/snp

International HapMap Project, http://hapmap.ncbi.nlm.nih.gov/

Online Mendelian Inheritance in Man, http://www.omim.org/

NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/

RefSeq, http://www.ncbi.nlm.nih.gov/RefSeq

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Figure Titles and Legends

Figure 1. Clinical Findings of PLACK Syndrome

(A) Family pedigree of individual 1. The arrow indicates individual 1 and W represents wild-type. (B) Individual 1 showed clinical features of generalized skin peeling, cheilitis, plantar keratoses on weight-bearing areas, blistering, leukonychia and knuckle pads with multiple hyperkeratotic micropapules. (C) Histopathology of skin biopsy from lower extremities of individual 1 demonstrated intraepidermal clefting with irregular acantholysis (hematoxylin-eosin staining, the scale bar represents 50 μm). (D) Similar clinical features were found in individual 2, including skin peeling, cheilitis, punctate keratoses of the soles, leukonychia and knuckle pads with hyperkeratotic micropapules.

Figure 2. Homozygous Mutations in *CAST* and Its Expression in the Epidermis

(A) Sequence chromatograms showing homozygous mutations c.607dup, c.424A>T and c.1750delG in the affected individuals. Arrows indicate the position of the mutation. (B) Homozygous frameshift mutation c.607dup-mediated mRNA decay of calpastatin in individual 1 is demonstrated by qRT-PCR compared to the normal control. The error bar represents the standard error of the mean (SEM). (C) Immunohistochemistry shows absent calpastatin expression in individual 1(right panel) compared to normal expression throughout the epidermis in the control (left panel). The scale bars represent 50 μm.

Figure 3. Decreased Expression of Filaggrin and Increased Keratinocyte Apoptosis in Lesional Skin

(A, B) Compared to the normal control (A), markedly reduced expression of filaggrin was noted in individual 1 (B). Scale bars represent 50 μm. (C, D) TUNEL assay demonstrated increased apoptotic level of keratinocytes in lesional skin from individual 1 (D) compared to the normal control (C). The scale bars represent 50 μm. (E) TEM of lesional skin from individual 1 showed expanded intercellular spaces (asterisks). (F) At higher magnification, chromatin condensation and margination were apparent.

Figure 4. CAST Knockdown Decreases Intercellular Adhesion and Increases Desmoplakin Expression

(A-C) HaCaT cells transfected with a pool of NTP siRNA (A) or CAST siRNA (B) were stained with an anti-calpastatin (green) antibody and DAPI (blue) as a nuclear marker. Total protein from HaCaT cell lysates after transfection with NTP siRNA (lane 1) or CAST siRNA (lane 2) were incubated with an anti-calpastatin antibody (C). GAPDH was used as loading control. A significant reduction in calpastatin protein levels can be observed in the CAST KD cells compared to NTP cells, both by immunocytochemistry and western blotting. (D-G) CAST KD and NTP cells were subjected to cyclic stretching for 0 hr (unstretched; D, F) and 4 hr (E, G) followed by staining with an anti-keratin 14 antibody. Stretching of the keratin filaments can be observed after 4 hr mechanical stress in the NTP cells (arrows) compared to

unstretched monolayers. In the CAST KD cells breakage of these filaments together with widening of the intercellular spaces was observed independent of mechanical stress (asterisks). (H, I) Immunohistochemistry with an anti-desmoplakin antibody on skin sections from individual 2 revealed an upregulation and change in localization of this protein from a membranous appearance (H) to a both membranous and cytoplasmic appearance (I) in comparison to control. The scale bars on all images represent 20 µm.

Figure 1
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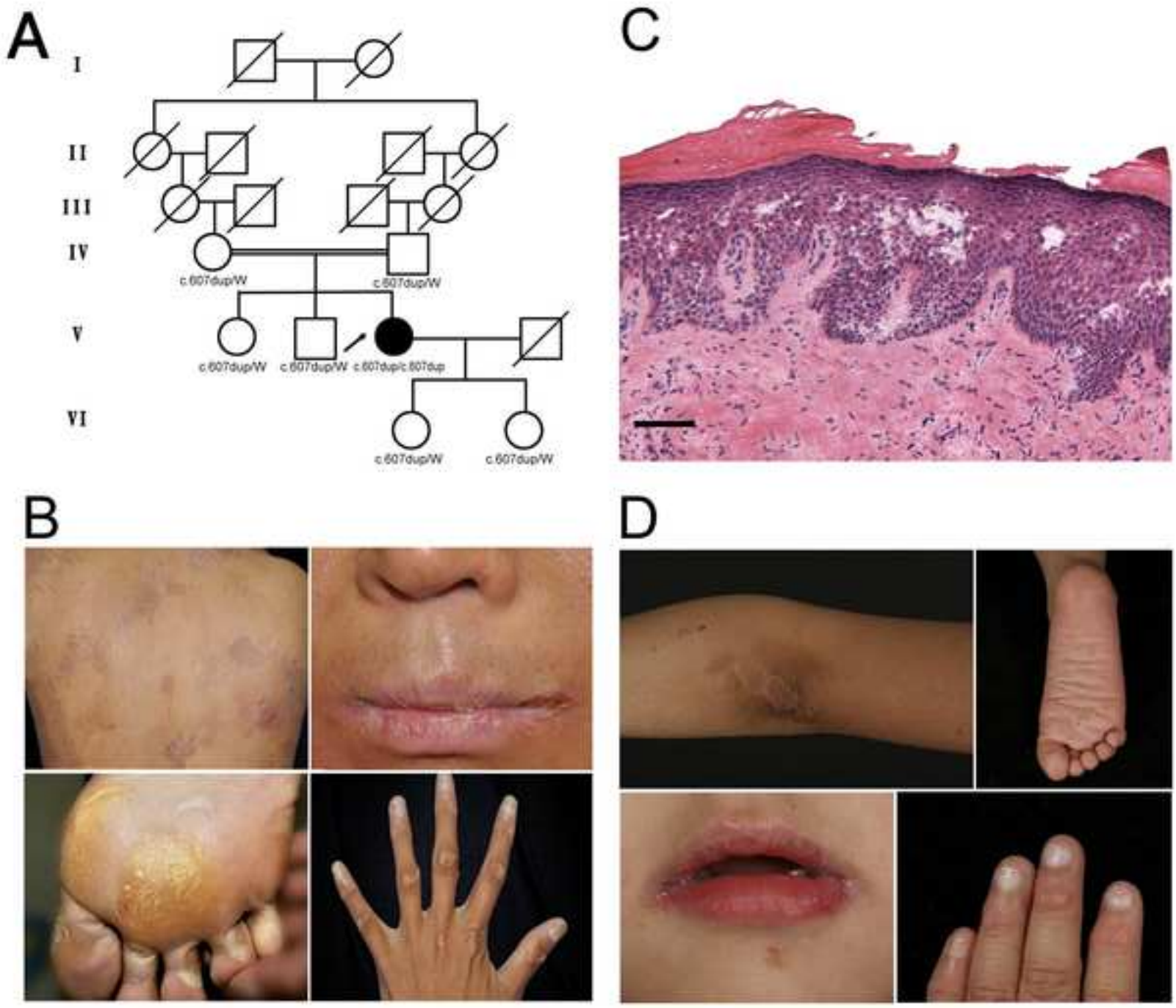

Figure 2
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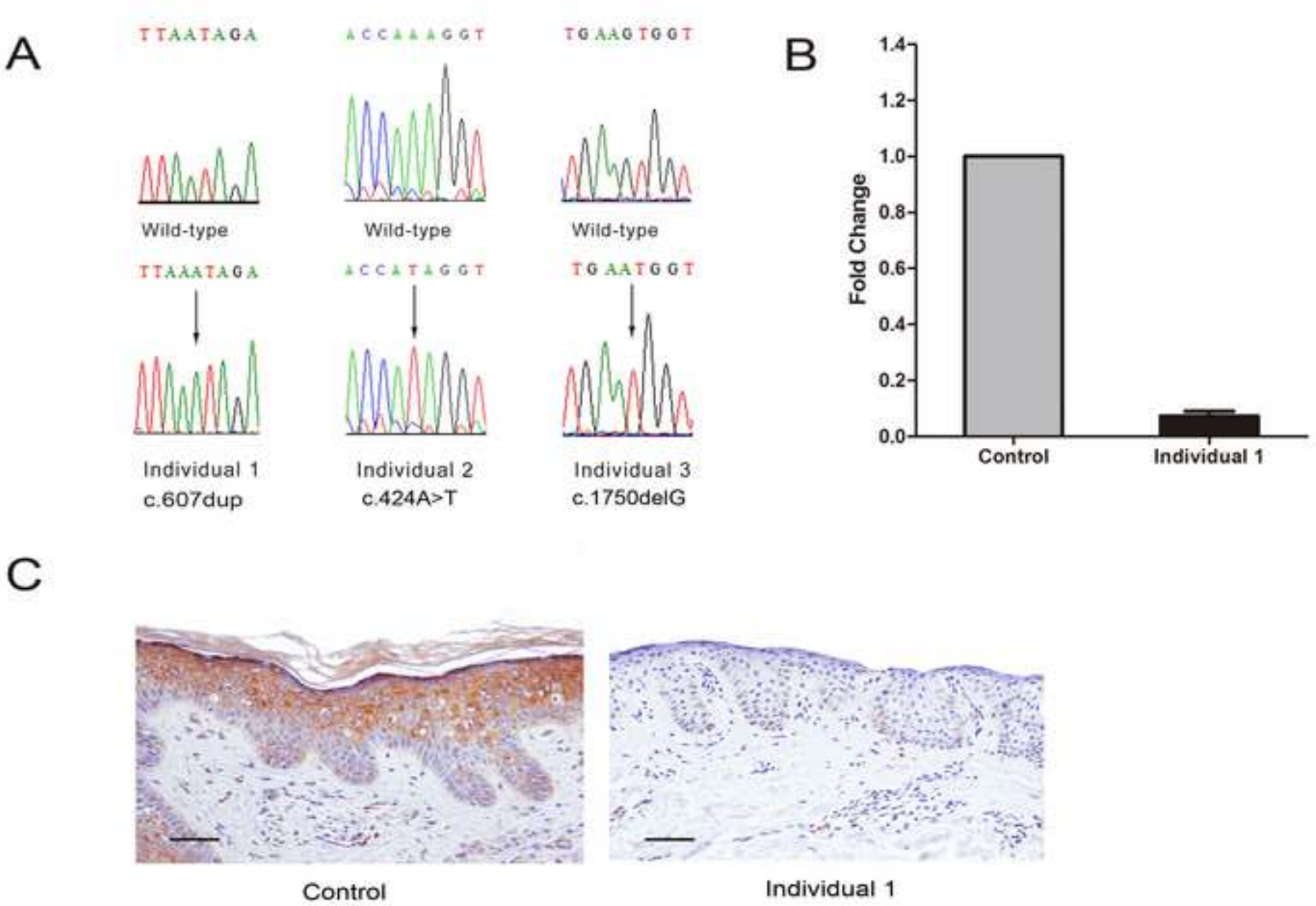

Figure 3
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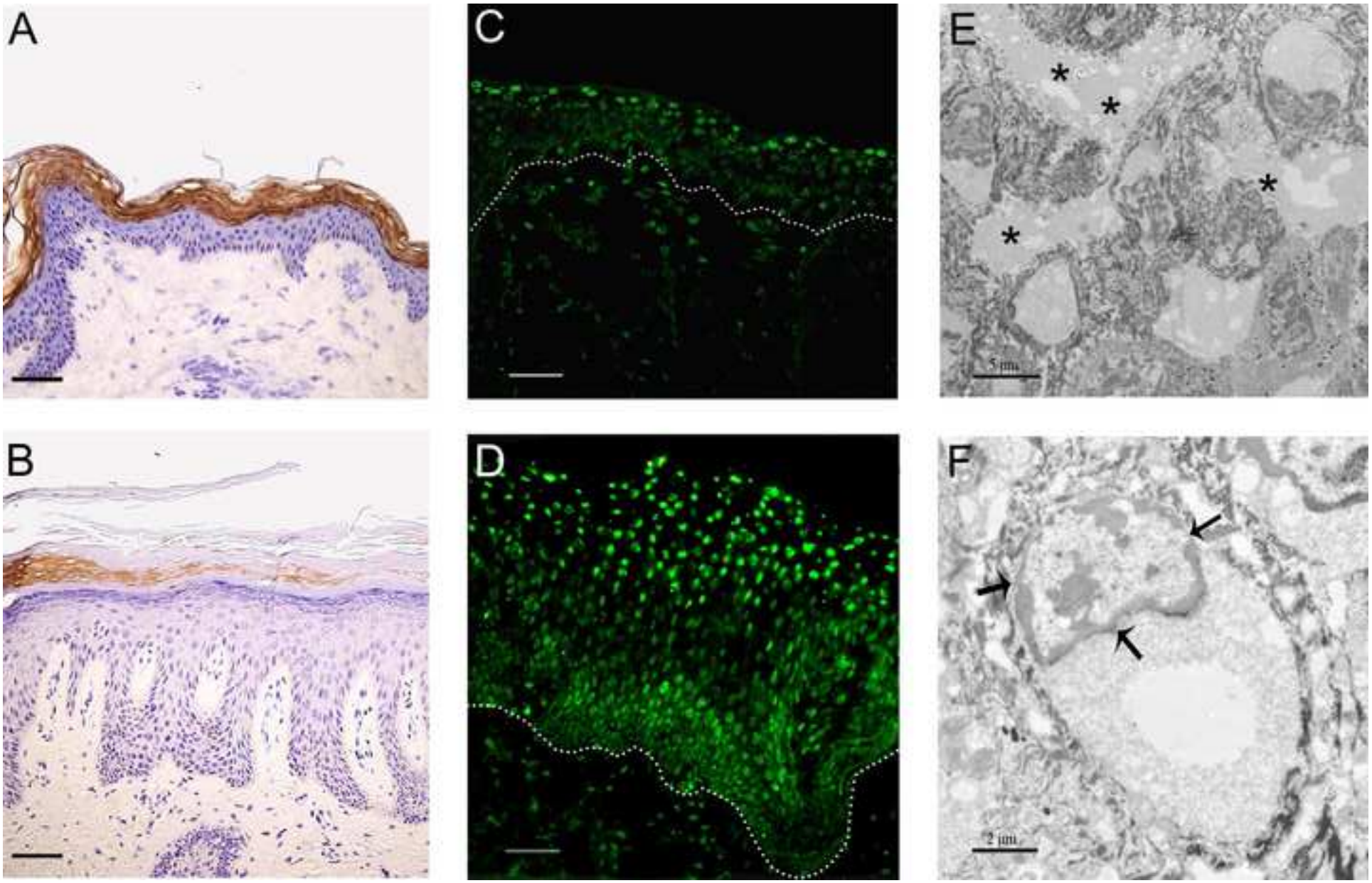

Figure 4

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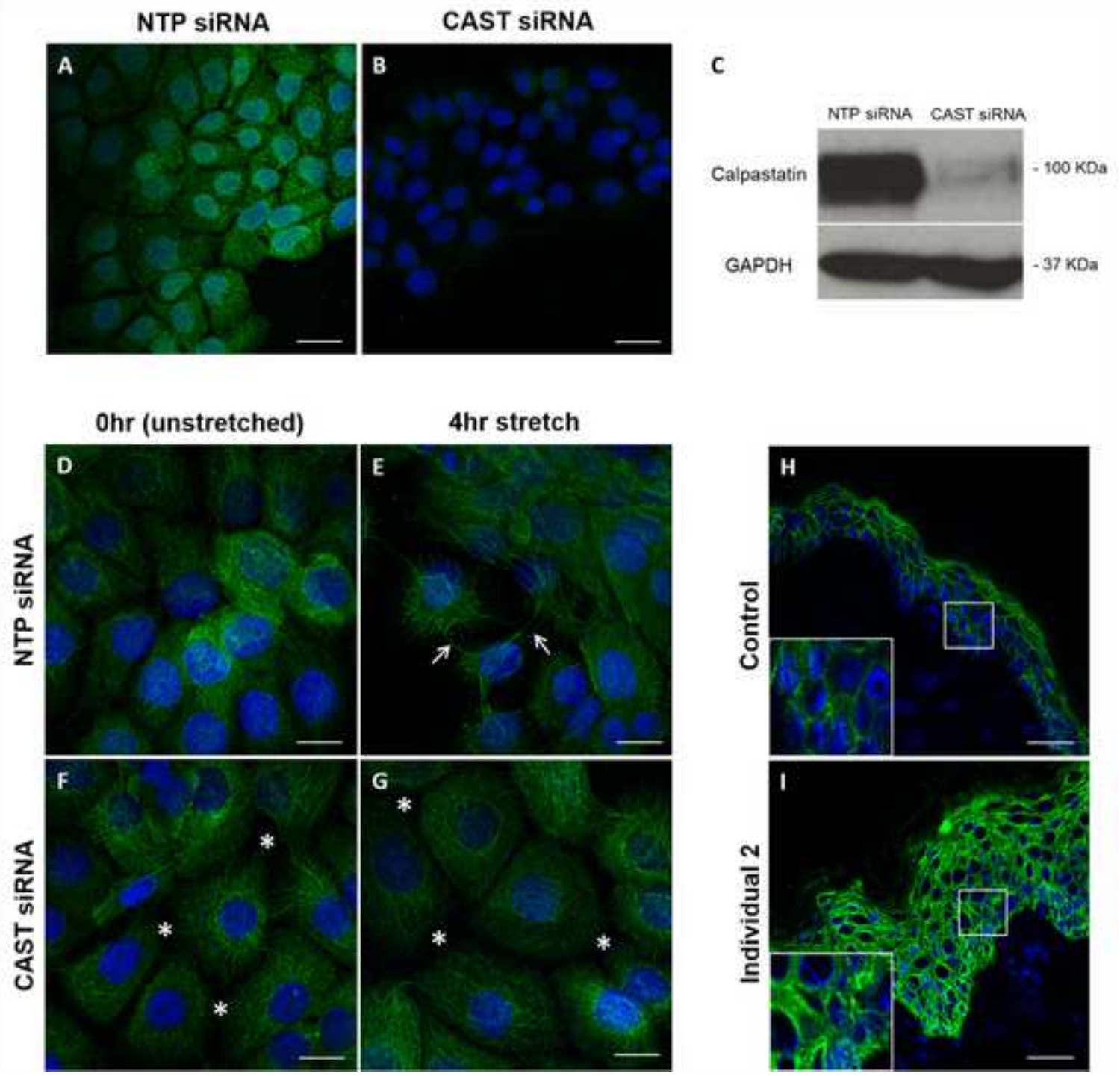

Loss-of-function mutations in *CAST* cause peeling skin, leukonychia, acral punctate keratoses, cheilitis and knuckle pads (PLACK) syndrome

Zhimiao Lin, Jiahui Zhao, Daniela Nitoiu, Claire A. Scott, Vincent Plagnol, Frances J. D. Smith, Neil J. Wilson, Christian Cole, Mary E. Schwartz, W. H. Irwin McLean, Huijun Wang, Cheng Feng, Lina Duo, Eray Yihui Zhou, Yali Ren, Lanlan Dai, Yulan Chen, Jianguo Zhang, Xun Xu, Edel A. O'Toole, David P. Kelsell and Yong Yang

Table S1. Primer Pairs for Confirmation of *CAST* Mutations

	Forward Primers (5'-3')	**Reverse Primers (5'-3')**
c.607dup	GCTTCTTGCCTGAATGTGGC	CCATGGCCTTATTTGCTCTCC
c.424A>T	AATTTTGGGGGAAGGATTTG	ATTGCTGGGCAGTAGGAGAA
c.1750delG	AGTTAAGTGATGGCATTGTGC	CATCTCGCTAAATCATCAGTC

Table S2. Primer Pair for qRT-PCR

	Forward Primers (5’-3’)	**Reverse Primers (5’-3’)**
CAST	CACAGTGCCAGATGATGCT	TCCTCAGACAAAGCATCCAGA

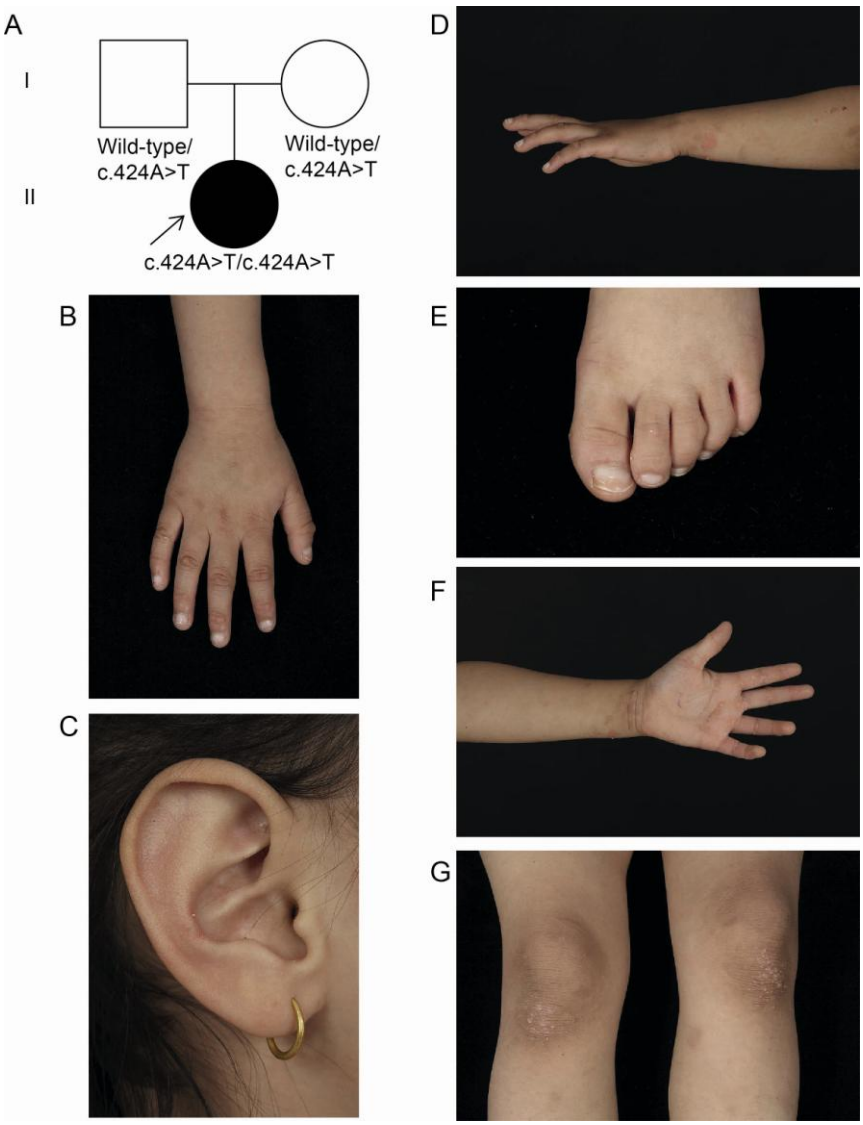

Figure S1. Further Details of the Clinical Phenotype of Individual 2.

(A) Pedigree of the family of individual 2 showing mutation segregation. The arrow indicates individual 2. (B) Overview of dorsum of hand showing knuckle pads on proximal and distal interphalangeal joints and leukonychia. (C) Scaly papule right ear antihelix. (D) Skin peeling with residual erythema left forearm. (E) Left foot showing leukonychia and papules with hyperkeratotic micropapules left second toe. (F) Punctate keratoses palm, most prominent along distal palmar creases. (G) Hyperkeratotic papules extensor surface of knees.

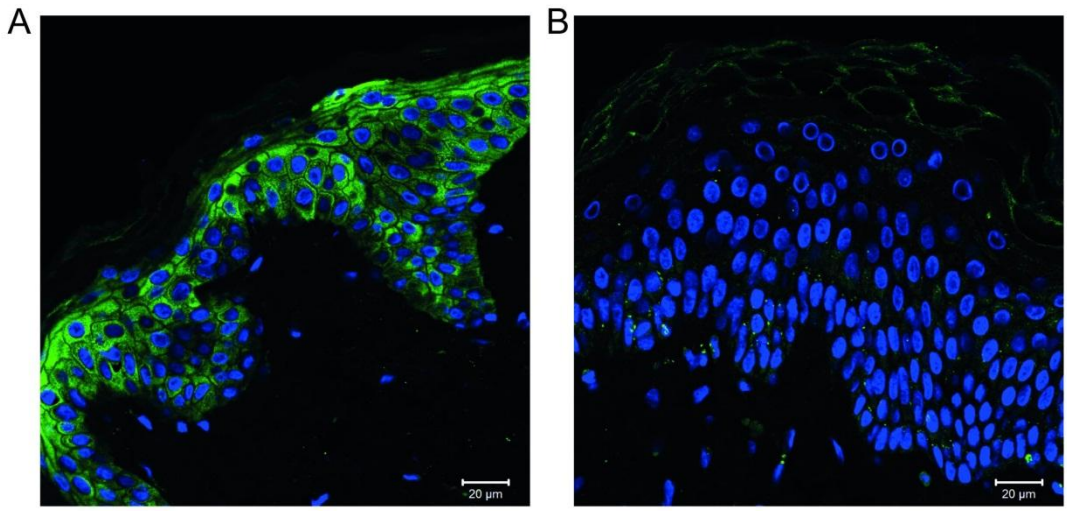

Figure S2. Immunohistochemical Staining of Calpastatin in the Epidermis of Individual 2 and the Normal Control

(A, B) Immunofluorescent staining of calpastatin (green) showed that calpastatin expression is reduced in individual 2 (B) compared to normal control (A). DAPI was used to stain the nuclei (blue).

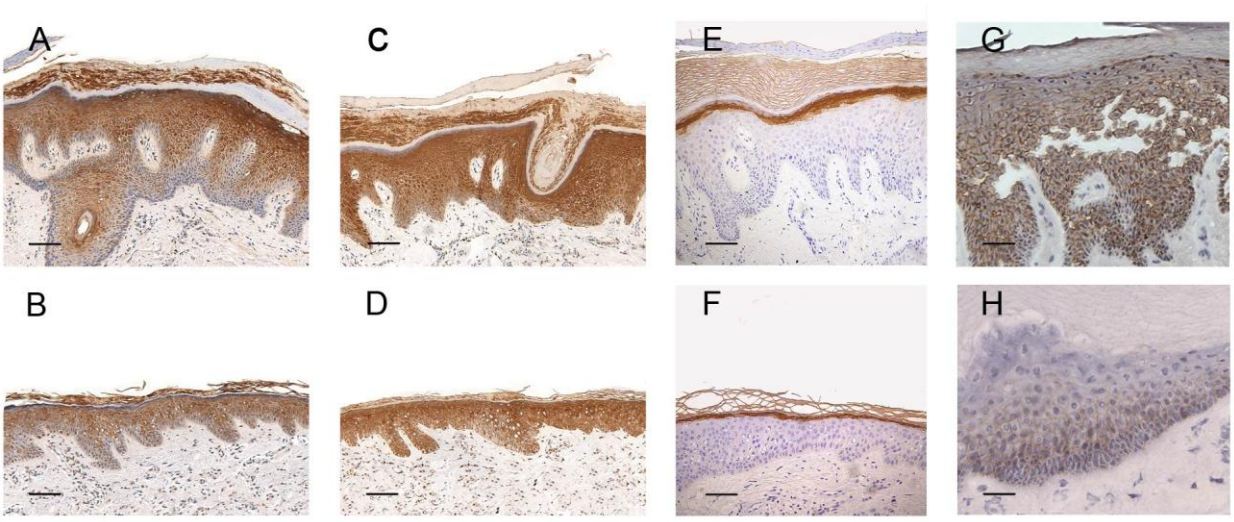

Figure S3. Immunohistochemical Staining of Terminal Differentiation Markers and Desmoplakin in the Epidermis of Individual 1 and the Normal Control

Keratin 1 (A), keratin 10 (C) and loricrin (E) of individual 1 showed normal distribution with slightly increased expression in the upper epidermis compared with the normal control (B, D, F). Desmoplakin expression was upregulated in individual 1 with an abnormal cytoplasmic localization (G) compared to the normal control (H). The scale bars on all images represent 50 µm.

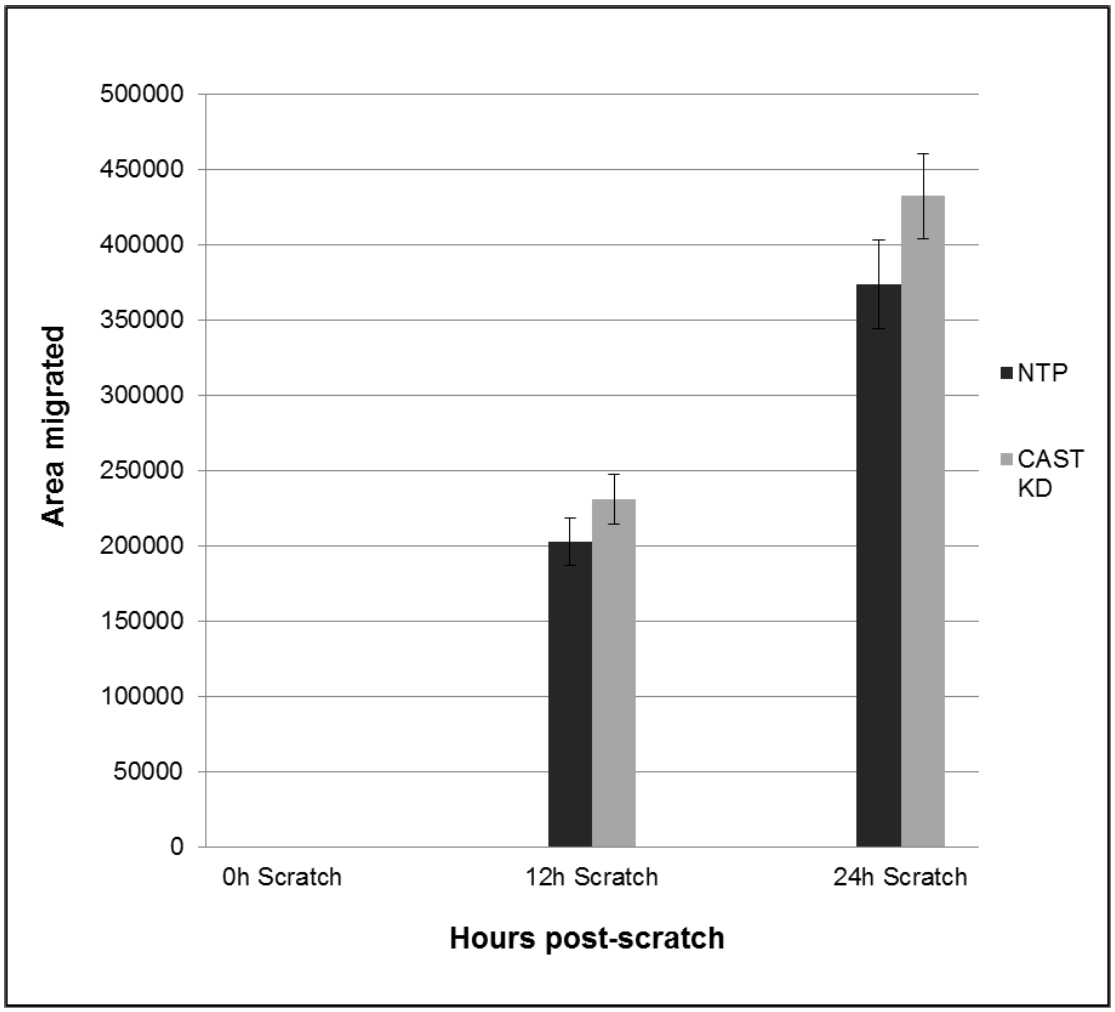

Figure S4. Scratch Assay

No significant difference was observed between NTP siRNA and CAST siRNA treated cells suggesting that there is no significant increase in cell migration and scratch closure (n=8 from 3 independent experiments).